

## A METHOD FOR THE CULTIVATION OF DISPERSED EPITHELIAL CELLS FROM MOUSE PALATAL MUCOSA<sup>1</sup>

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**Abstract.** A cell dispersion method is described for establishing cultures of mouse palatal epithelial cells which are viable in primary cultures and subcultures for extended periods of time. The cells *in vitro* mainly displayed the normal morphologic characteristics of palatal epithelial cells. As demonstrated by labelling the cells with tritiated thymidine, the monolayers of epithelial cells possess a high rate of DNA synthesis. Karyotypes of the cultured cells show a high percentage of normal displays of chromosomal numbers typical of the mouse strain employed. The number of spontaneous aberrations observed is in agreement with previous investigations using other methods.

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Cultivation of adult oral epithelium *in vitro* for limited periods, mostly as organ or explant cultures, has been reported. Virtually without exception, either fibroblastic overgrowth of cultures (Rose *et al* 1967, Bracho *et al* 1970) or keratinization of cultured epithelial cells was observed (Porter 1960, Neiders and Weiss 1970). Milnek and Buchner (1975) recently described a simple and reliable method for the cultivation of human gingiva for epithelial outgrowth in which the fibroblastic emigration over a 3 week period was insignificant in comparison to the large epithelial outgrowth. Numerous attempts to cultivate disaggregated epithelial cells have been made in addition to the studies of epithelium grown in organ or explant cultures (Smulow and Glickman 1966, Karasek 1975, Rheinwald and Green 1975). In general, such efforts resulted in monolayers with greater growth but were usually maintained for very limited periods in primary culture. The present study is concerned with a method for the establishment of cultures of adult mouse palatal epithelium

which are viable in primary cultures and in subcultures for extended periods of time, for studies of the morphology and karyotypes of the cells.

### MATERIALS AND METHODS

**Cell Culture Technique.** Swiss albino male mice (5-6 weeks old) were sacrificed by cervical dislocation. Incisions were made at the posterior aspect of the hard palate and at the palatal margins of the teeth. The palatal mucosa was stripped from the hard palate and washed several times in Hanks' Balanced Salt Solution (HBSS). Samples consisted of pooled amounts of palatal epithelium from several mice cut into small fragments (~1mm<sup>2</sup>) and washed twice with HBSS.

The minced epithelial fragments were treated with a 0.1% solution of Pronase in HBSS for 30 to 60 minutes at 37°C in a trypsinizing flask until a slightly feathered appearance was noted to indicate intercellular separation. The sample was then centrifuged for 10 minutes at 131 x g and the resulting pellet was washed twice with HBSS. Mechanical dispersion of the pellet was effected with a magnetic stirring bar for 30 minutes. The cell suspension was then decanted into centrifuge tubes and centrifuged at 131 x g for 15 minutes, then resuspended in sufficient growth medium to provide a concentration of approximately 250,000 cells/ml. The viability of the final cell suspension was determined by the trypan blue dye exclusion test (Merchant *et al* 1964).

The cells were grown in Eagle's Basal Medium (BME) supplemented with 10% fetal calf serum (previously decomplexed by heating to 56°C for 40 minutes), 1% *l*-glutamine and a 1% antibiotic-antimycotic mixture containing 10,000 units/ml of penicillin, 35 µg/*l* fungizone, and 10,000 µg/ml streptomycin. The cell sus-

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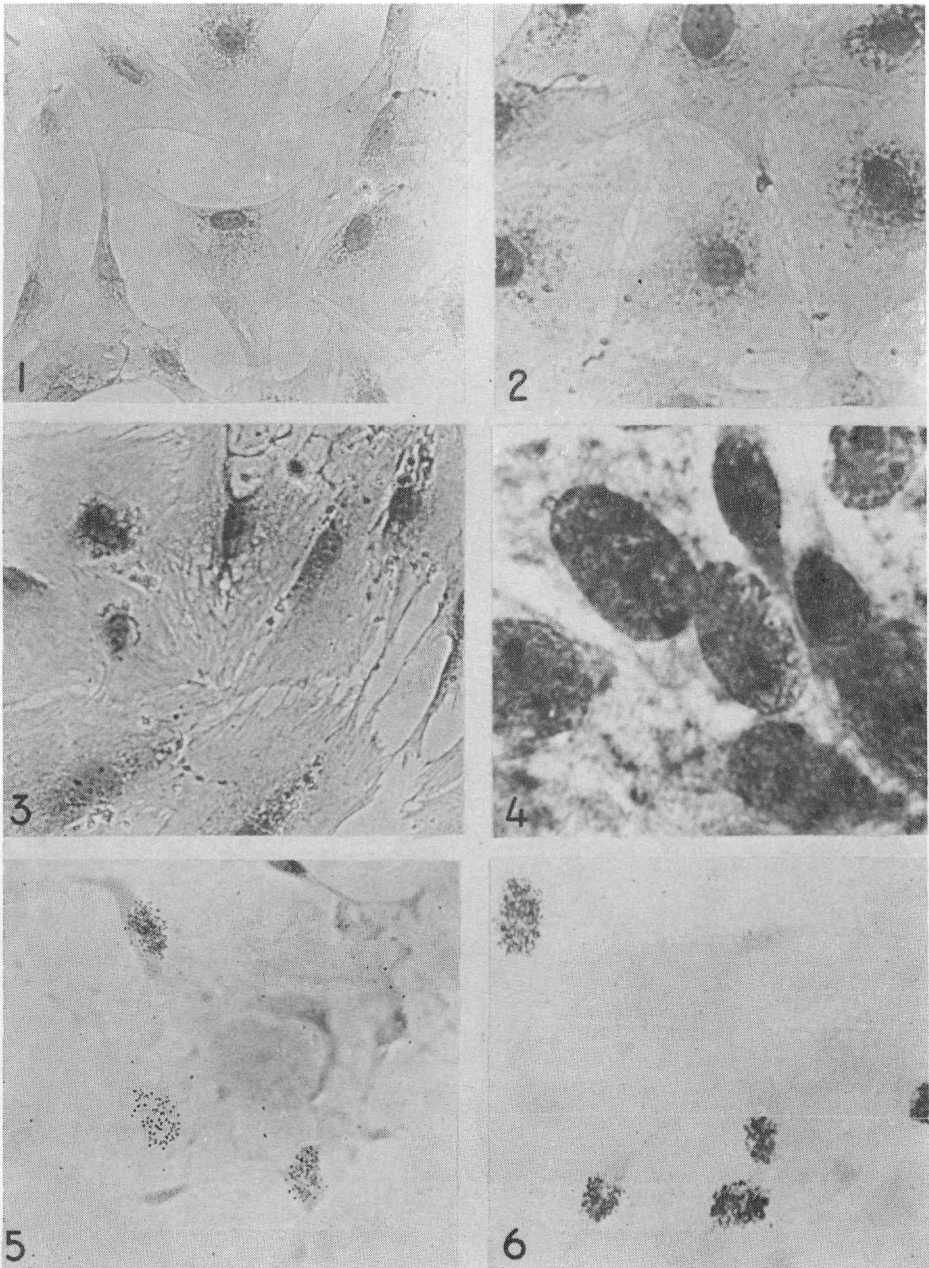


FIGURE 1. Monolayer of normal palatal epithelial cells grown *in vitro* for 10 days. Original magnification  $\times 500$ .

FIGURE 2. Higher power magnification of monolayer of normal palatal epithelial cells grown *in vitro* for 10 days. Original magnification  $\times 800$ .

FIGURE 3. Primary culture of normal palatal epithelial cell monolayer grown *in vitro* for 28 days. Original magnification  $\times 500$ .

FIGURE 4. Subculture of palatal epithelial cell monolayer grown *in vitro* for 72 days. Phase contrast microscopy. Original magnification  $\times 1200$ .

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FIGURE 5. Monolayer of normal palatal epithelial cells grown *in vitro* for 10 days, treated with tritiated thymidine for 1 hour and exposed for 3 days. Note the labelled cells in contrast to the number of unlabelled cells. Phase contrast microscopy. Original magnification  $\times 250$ .

FIGURE 6. Monolayer of normal palatal epithelial cells grown *in vitro* for 10 days, treated with tritiated thymidine for 1 hour and exposed for 7 days. Especially note the large number of labelled cells. Phase contrast microscopy. Original magnification  $\times 250$ .

pension (0.5 ml) was seeded into a series of plastic tissue culture flasks containing 5 ml of basal medium and 2 days later 2 to 3 ml of additional medium was added to maintain the buffering capacity. After the first few days of culture, the indicator showed increased alkalinity and readjustment was accomplished by introducing a gas mixture of 5% CO<sub>2</sub> enriched air for a short period every 6 hours, or by adding 5 ml of BME supplemented with 25 mM Hepes buffer (with Hank's salts plus the fetal calf serum, *L*-glutamine, and the antibiotic-antimycotic mixture). Use of the latter method revealed superior buffering capacity and better growth in comparison to the use of the gas mixture. Cultures were incubated at 37 °C and the medium was changed after 4 days (except as detailed above) with successive changes of the growth medium conducted every 2 days thereafter.

Subculture was performed on the 10th to 12th day, after the initial culture displayed a monolayer growth throughout the flask. The medium was decanted from the flask and the culture washed with a few ml of HBSS. Two to three ml trypsin (0.1%) in HBSS (Gibco) was added and the cultures were examined until the monolayer detached. A few ml of medium (with fetal calf serum) was then added and the suspended cells were dispersed by gentle shaking. The suspension was then pipetted into 2 new flasks. After 10 to 12 days, when the new confluent monolayer was distributed over the entire bottom of the flask, subculture was repeated again.

**Thymidine Uptake Studies.** The time of the S phase of the cells of primary cultures was determined through autoradiography using tritiated thymidine (<sup>3</sup>H-TDR). Ten day control cultures were established in Lab-Tek flasks. One ml of <sup>3</sup>H-TDR containing 1.0  $\mu$ Ci/ml (Sp. Act. = 1.9 Ci/m mol) was added to 4 ml of BME in the culture flask and incubated for 1 hour. The medium was then decanted and the cell culture washed with HBSS 4 times and a few milliliters of 10% neutral buffered formalin was added to fix the cell layer. After 30 minutes, the fixative was decanted and the cells washed twice with sterile distilled water. The flasks were then separated from their glass base (which consists of a glass microscope slide), and warmed prior to coating with NTB3 autoradiographic film emulsion. The coated slides were then air dried (in the dark), after which they were placed in a light tight slide box with Drierite, sealed and refrigerated. The optimum exposure period of the autoradiographs was determined by serially developing sample slides in Kodak D19 developer for 5 minutes at 20°C. After fixing and washing, the underlying sections were stained with hematoxylin. The 3 and 7 day exposures demonstrated significant silver grains, indicative of

<sup>3</sup>H-TDR uptake in the cells in S phase.

**Chromosome Preparation.** After establishment of the monolayer in the primary cultures (on day 10) chromosome preparations were made as follows:

1. Colcemid solution 0.02 ml for each ml of BME in the culture (final conc. 0.6  $\mu$ g/ml), was added to the medium and the culture was reincubated for 4 hours at 37°C. The growth medium containing the Colcemid was decanted but not discarded.
2. To the culture flask 2-3 ml of HBSS was added, followed by a 5 min. reincubation.
3. The cultures were then vigorously shaken and the supernatant decanted and added to the original decanted growth medium. The remaining adherent cells were removed mechanically with a rubber policeman and the pooled decanted material was centrifuged for 10 minutes at 131 x g. The supernatant was removed and discarded.
4. A hypotonic solution of 1% sodium citrate was then added to the pellet and the compacted cells immediately dispersed with a Pasteur pipette, then permitted to stand for 10 minutes.
5. A few drops of freshly prepared ice-cold fixative (3:1 methanol-glacial acetic acid) was added. After 2 min the preparation was centrifuged for 4 min at 98 x g following which the supernatant was removed and 5-6 ml of additional fixative added to the cells which were allowed to stand for 20 minutes without mixing or dispersing.
6. After this period, the cells were dispersed with a Pasteur pipette, and centrifuged for 4 minutes at 98 x g. The sample was allowed to stand for 5-10 minutes before the supernatant was poured off and a new cold fixative added to the pellet for 10 minutes.
7. The supernatant (except for appx. 0.5 ml above the pellet) was removed and the tubes chilled on ice for 10 minutes. Thoroughly cleaned and chilled slides were used to make the cell preparations.
8. The chilled cell suspension was dropped onto the chilled slides and the preparations were immediately dried by passing the slides over a very low flame. The preparation was stained with Giemsa for 7-10 minutes and washed twice with sodium phosphate buffer (pH 7.1), and air dried. Data were statistically evaluated using Student's t test and a confidence level of  $P < 0.05$ .

## EXPERIMENTAL RESULTS

We have described a technique for obtaining adult mouse palatal epithelium cultures which are viable in primary cultures and subcultures for extended periods

of time (up to 72 days). In contrast with previously reported work, this technique employs a cell dispersion technique. Under optimal conditions, the total number of cells per specimen was approximately  $1.25 \times 10^5$  and consisted mostly of single cells. The trypan blue exclusion method demonstrated a viability level of approximately 70% of the initially dispersed cells. In terms of each culture preparation, this means that a minimum of approximately 87,500 viable cells were used. After 24 hours in culture, approximately 500 cells were observed to be attached to the tissue culture flask.

Figures 1 and 2 show photomicrographs of a typical sheet of epithelial cells proliferating from the original inoculum after 10 days of culture. These cells display rounded nuclei and cytoplasmic morphology typical of cultured epithelial cells and show the phenomenon of contact inhibition limiting cell overgrowth so as to grow in a monolayer fashion. Figure 3 shows a typical representation of the growth of monolayers in the cultures over a period of 28 days. The monolayer configuration is maintained, and the cells manifest the morphology typical of primary cultures. Monolayers were found to persist up to 40 days beyond which period viable cells were seen to grow over the remains of necrotic cells. We have successfully maintained subcultures up to 72 days at which time some of the nuclei became hyperchromatic with the cytoplasm displaying a hypergranulation characteristic of degenerating cells (figure 4).

Monolayers of normal 10 day old primary cultures of palatal epithelial cells treated with  $^3\text{H}$ -TDR for 1 hour and exposed for 3 or 7 days have a high rate of DNA synthesis. In a total population of 2,221 cells counted, the percentage of labelled cells  $\pm$  S.E.M. has been estimated to be  $20.9 \pm 1.5$ . Figures 5 and 6 show typical photomicrographs of 10 day old primary culture monolayers of normal palatal cells treated with  $^3\text{H}$ -TDR for 1 hour after 3 and 7 days of exposure. The large number of labelled epithelial cells present in each of the monolayers reinforce the indicated viability of these cells in our tissue culture system.

Karyotypes of the primary cultures were prepared 10 days after culture initiation. Figure 7 shows a typical metaphase spread in a 10 day old normal culture revealing the presence of 20 pairs of chromosomes. It should be noted that mitotic spreads from subcultures up to 28 days also showed approximately 70% of the cells to be normal epithelial cells with the normal number of chromosomes typical of figure 7.

In 4 experiments dealing with normal 10 day old control primary cultures, an examination of chromosome spreads showed the presence of 184 metaphases. The mean number of diploid cells  $\pm$  SE per group was  $37.5 \pm 7$  and the number of heteroploids constituted  $8.5 \pm 2$  of the population of cells examined. A heteroploid display of chromosomes from epithelial cells in metaphase from 10 day old primary cultures is shown in figure 8. Of all the metaphase cells examined only 7 cells out of 184 showed a total of 8 aberrations (4.4%). The types of aberrations seen included 3 chromatid breaks, 3 achromatic lesions (gaps), and 2 acentric chromosomes.

#### DISCUSSION

Pronase was used to obtain initial suspensions of palatal tissue consisting chiefly of epithelial cells. Neiders and Weiss (1970) reported an epidermal-dermal separation on enzymatic treatment of human gingiva and that longer incubation with Pronase produced epithelial and fibroblastic cultures. We assume in our system that Pronase initially acts to separate the epithelium from the underlying connective tissue, and that the intercellular matrix of the epithelium is preferentially attacked by the enzyme. Once the intercellular attachment is disrupted by the Pronase, mechanical disruption is sufficient to give a suspension consisting mainly of single epithelial cells. Since these cultures have few fibroblasts in them to start with, the fibroblastic overgrowth is eliminated. When such epithelial cells are grown in monolayers, apparently keratinization of these cells is prevented. The fact that only a small percentage of cells attach to the tissue culture flasks and grow suggests that only the germinativum cells

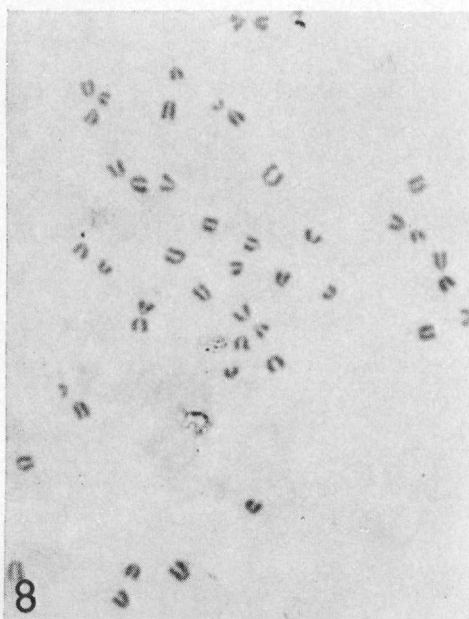
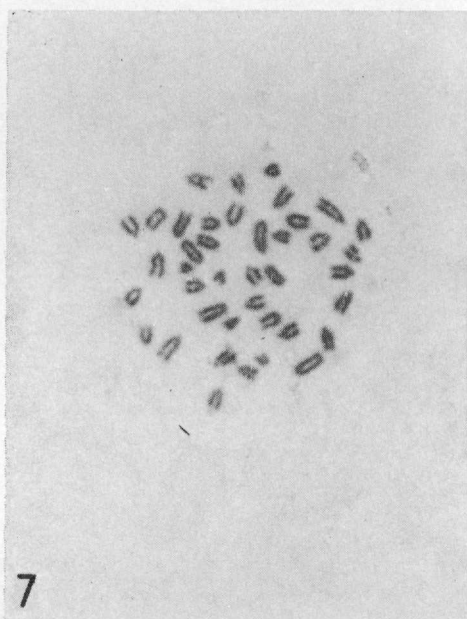


FIGURE 7. A typical preparation showing the normal number of chromosomes of an epithelial cell in metaphase from a 10 day old normal culture. Original magnification  $\times 400$ .

FIGURE 8. A heteroploid display of chromosomes of palatal epithelial cells in metaphase from 10 day old cultures. Original magnification  $\times 400$ .

are involved. This appears to be in accord with the report of Constable (1972) that the remainder of the cells from the superficial layers apparently lose their potential to adapt and proliferate.

Buffering appears to be a crucial factor for ensuring good growth *in vitro*. The efficiency of the cell dispersion technique employed is shown by the maintenance of the rounded nucleus and cytoplasmic morphology of the epithelial cells with confluent growth in both primary cultures and subcultures. Pilot electron microscopic studies of control cultures showed the typical ultrastructure of active monolayered epithelial cells and desmosomes with tonofilaments (generally considered a good marker for the presence of epithelial cells). It was noted that collagen fibrils were not apparent in any of the sections examined, although this may be due to the fact that they are generally absent in monolayered cultures. The viability of the cultures was demonstrated by the high labelling index noted with tritiated thymidine. Future studies utilizing labelling tech-

niques at various stages of the cultures would be valuable to elucidate the growth patterns of the epithelial cells employed in our system.

Our chromosome spreads of the cultures were prepared in the conventional manner from Swiss albino mouse tissue which normally has 20 pairs of chromosomes. Our cultured epithelial cells in metaphase showed the number of chromosomes typical of the mouse strain employed. The number of heteroploids we observed in normal cultures appeared to be within the expected percentage of spontaneous aberrations (Elkin and Whitmore 1967). Apart from heteroploidy, the types of spontaneous aberrations noted in our cultures appeared to be in accord with Elkin and Whitmore's (1967) findings that the most prevalent type seen are of the chromatid variety.

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